MOLECULAR CATALYSIS AND INTERACTIONS IN AQUEOUS SOLUTIONS.

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INTRODUCTION

The present studies have been concerned with mechanisms of hydrolysis of a phosphonate ester which is shown to behave in a manner thought to be similar to the process responsible for the "ageing" effect observed for cholinesterase inhibited by phosphorylating agents. The particular system investigated was the ester formed through interaction of Sarin (isopropyl methyl phosphonofluoridate) with 3-nitrocatechol. The end product and the pH profile exhibited by the hydrolyzing system closely resembles the process apparently responsible for the irreversibility gradually produced in the freshly inhibited enzymes.

It is now firmly established that the inhibition of cholinesterases and related enzymes by organophosphorus compounds is due to dialkylphosphorylation at or near the active site of the enzyme molecule. The evidence[1] for direct phosphorylation is both stoichiometric and kinetic and may be represented (when EH is the uninhibited active form of cholinesterase) by:

$$R'(RO)PO(X) + EH \longrightarrow R'(RO)POE + HX$$

where R' = aryl, alkyl, aryloxy or alkoxy.

The activity of the phosphorylated enzyme apparently can only be restored through a dephosphorylation process.

The reversal of inhibition involves a hydrolysis which yields (in the case of dialkyl phosphate inhibition) a dialkyl phosphoric acid and the active enzyme. If the hydrolysis is slow, the inhibition appears to be substantially irreversible.

The rate of reactivation of the phosphorylated enzyme has been shown[2] to be independent of the acidic (X) group of the original inhibitor. Rather, it has been reported to be dependent on other variables such as the nature of the basic (alkyl) substituents on the phosphorus,[3] the structure and chemical properties of the reactivating agents,[4] the pH of the reaction,[5] and the time of storage.[6] The latter dependency indicated that the longer the inhibitor was in contact with the enzyme, the less the fraction of inhibited enzyme that could be recovered by subsequent treatment with the reactivating agent. Thus, two kinds of reactivations were subsequently reported[7]: a) one in which the reactivation process is time dependent and resembles a normal second order reaction, and b) another in which, after an initial rapid reactivation, little further change occurs in the degree of reactivation of the inhibited enzyme. This second conversion type was first observed by Hobbiger[8] in 1955 who noted that the extent of reactivation of organophosphate serum cholinesterase that could be dephosphorylated by nicotinhydroxamic acid methiodide depended on how long the

enzyme and inhibitor had been left in contact. The results indicated that a chemical reaction had taken place in which the unstable form of the phosphorylated enzyme, which can be reactivated by nucleophilic compounds, is converted to a stable form which cannot be. This phenomenon was termed "ageing".

Various theories were proposed[9] as to the character of this reaction. It is now generally accepted that "ageing" in the case of cholinesterases is caused by dealkylation of the dialkylphosphorylated (DP) enzyme to the more stable mono-phosphorylated (MP) form. Moreover, the pH dependency of this "ageing" process ~~has been shown to be~~ shows that the reaction is acid catalyzed.[10] Berends and his co-workers[11] proved that di-isopropyl phosphorofluoridate-(DFP)-inhibited-pseudo-cholinesterase, when "aged," converts from the DP into the MP enzyme accompanied by the appearance of free isopropanol.

Recent studies confirm the acid catalysis of the "ageing" process[12] and in most cases support the dealkylation theory.[13] It should be emphasized that much of the data on such inhibited enzyme reactivations can not be justifiably applied to other systems, due to individual enzyme specificities, inhibitor variations and other reaction variables. The "ageing" phenomenon, however, can help to explain many of the mysterious storage effects experienced in earlier enzyme

reactivation studies.

In an attempt to accelerate the rate of reactivation of phosphorylated enzymes, various nucleophilic reagents were studied both from a standpoint of a) catalyzing the hydrolysis of the original inhibiting organophosphorus compound[14] and, b) reactivating the inhibited phosphorylated enzyme to the free active form.[4] In particular, studies determining the effects of several catalytic species on the hydrolysis of Sarin and DFP attracted the attention of several investigators.[15]

The discovery[16] that catechol and its derivatives reacted with DFP and Sarin at considerable speed and good yield gave indication that the enzymes which are inactivated by these compounds may be chemically related to aromatic ortho-dihydroxy structures. Epstein, Jandorf and others[17] studied the kinetics of the reaction of Sarin with phenols and catechols and showed that the enhanced activity of catechols toward Sarin, as compared to phenols, strongly suggested participation of the undissociated o-hydroxyl group. Russo[18] studied the reaction of Sarin with various phenolate and catecholate derivatives. He showed that the reaction with catechol and the subsequent hydrolysis of the Sarin catecholate ester underwent a rather unexpected and facile hydrolytic cleavage, strongly suggesting some form

reactivation studies.

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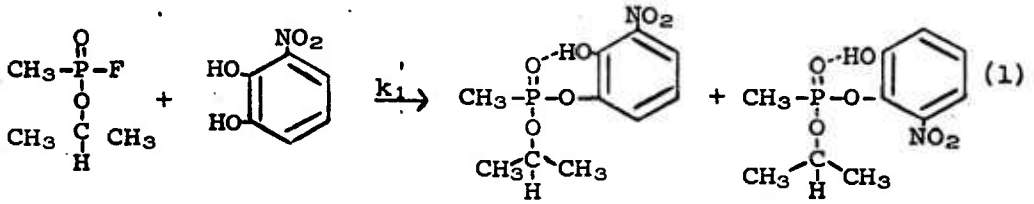
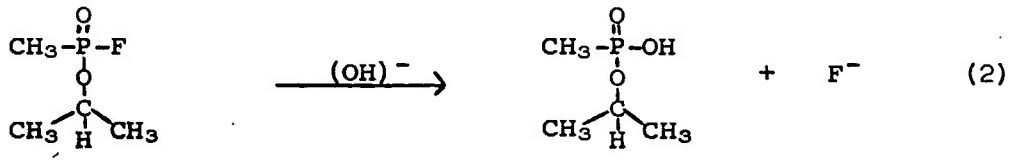
of Sarin under conditions favoring intramolecular catalysis of its ester. The study was also conducted at several temperatures to determine the energetics of the reaction.

THEORETICAL CONSIDERATIONS OF THE CHEMISTRY AND MECHANISMS OF THE SYSTEM

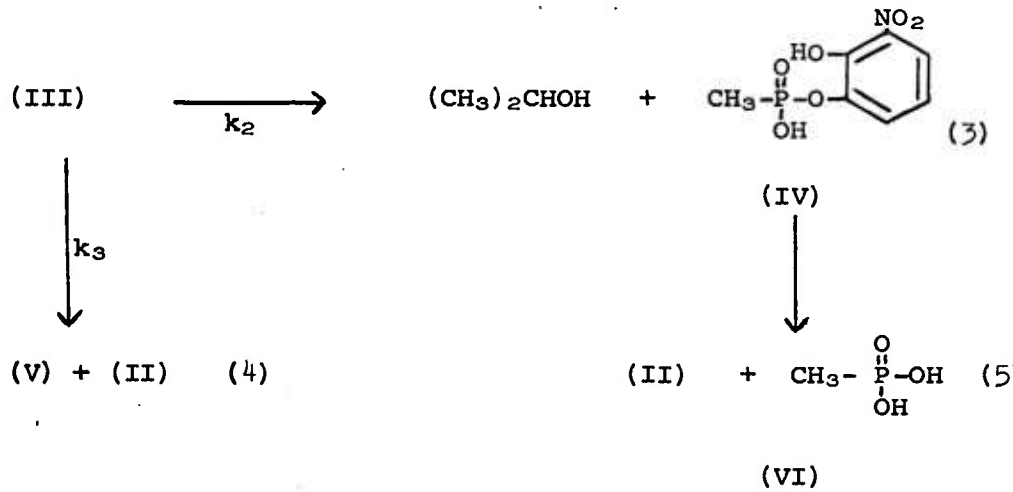
The reaction of Sarin (I) with nitrocatechol (II) can be expected to yield two possible products which would be position isomers (III) and (III') as shown represented (eq. 1) below in the form of a schematic reaction diagram. On the basis of earlier work, the phenolate reaction, as also shown in the outline, has been observed to be markedly faster than the alkaline cleavage (k_s) of Sarin itself[20] (eq. 2). In the present study, only the (III) form of the ester appeared to build up in concentrations of any significance. This ester can be further hydrolyzed to yield products which appear to depend on the nature of the hydrolytic cleavage. Cleavage of isopropyl ester linkage would yield the ester (IV) and isopropanol (eq. 3), whereas cleavage of the 3-nitrocatechol moiety would yield acid (V) and 3-nitrocatechol (eq. 4). Although compound (V) is known to be stable, compound (IV) may be subject to degradation to phosphonic acid (VI) and 3-nitrocatechol (eq. 5). Alternate syntheses of these compounds are shown in equations 6 - 9. Thus, the reaction scheme of equations 1 - 9 (with the respective product designations) will be used as a basis for further discussion of the system studied.



(I) (II) (III) (III') (1)



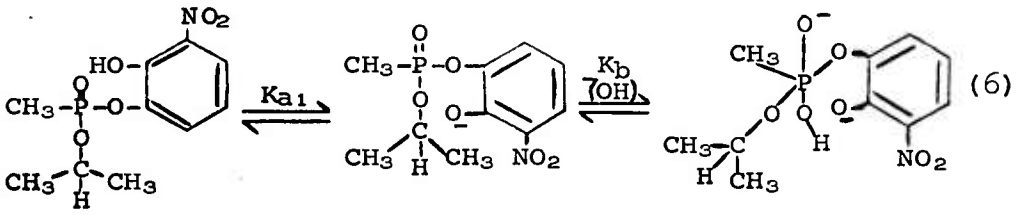
(I) (V) (2)



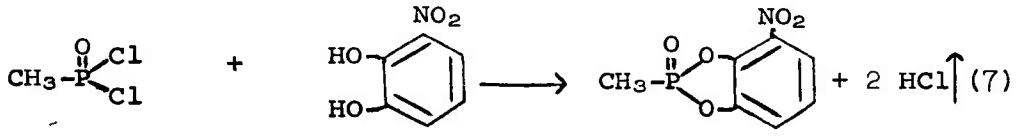
(III) (V) + (II) (4)

(III) (IV) (5)

(VI)



(III) (III$^-$) (III$^=$)



(VII) (VIII)

(VIII) $\xrightarrow[(CH_3)_2CHOH]{\text{anhydrous}}$ (III) (8)

(VIII) $\xrightarrow{HOH}$ (IV) (9)

Kinetic Derivation of k_1.- The concentration of 3-nitrocatechol can be kept in excess of Sarin, so that equation 1 becomes essentially pseudo first order with respect to Sarin, $k_1 = k_1'$ (II). In the consecutive reaction (I) $\xrightarrow{k_1}$ (III) $\xrightarrow{k_2}$ (IV) the value of $k_1 \gg k_2$ and thus, by conventional treatment [21] the kinetic rate expression can be derived showing that:

$$\frac{d(IV)}{dt} = k_2 (III) = \frac{k_2 k_1}{k_2 - k_1} (I)_o \left[e^{-k_1 t} - e^{-k_2 t} \right]$$

and

$$(IV) = (I)_o \left[1 + \frac{1}{k_1 - k_2} (k_2 e^{-k_1 t} - k_1 e^{-k_2 t}) \right]$$

Since $k_1 \gg k_2$ the expression reduces to

$$(IV) = (I)_o \left[1 - e^{-k_2 t} \right] \sim (I)_o k_2 t$$

therefore $(IV) = k_2' t$ if $k_2' = k_2 (I)_o$ where $(I)_o$ is the initial concentration of Sarin used. Initially, a lag time is observed and one can easily derive, when (IV) is extrapolated to zero (Fig. 2), a value of $t = \tau$ where $\tau = \frac{1}{k_1}$. Thus k_1 becomes the pseudo first order rate constant for equation 1 and k_2' is the observed pseudo rate constant for the overall reaction, equations 1 and 3.

Kinetic Derivation of k_2.- The development of the kinetic determinations of k_2 and k_3 can be based on the existence of isopropyl 3-nitro-o-hydroxy-phenyl methyl phosphonate (III) in three forms (eq. 6). The validity of this assump-

tion will be shown in later discussion. Based on equation 2 the kinetic relationship can be shown as

$$\text{rate} = \frac{-d\,(II)_T}{dt} = k_2(III)$$

where (III) is the amount of undissociated species present and $(III)_T$ is the total amount of ester where

$$(III)_T \doteq (III) + (III^-).$$

$$\text{Since } K_{a1} = \frac{(III^-)\,(H^+)}{(III)} = \frac{\left[(III)_T - (III^-)\right](H^+)}{(III)}$$

$$\text{and, rearranging, } (III) = \left[\frac{(III)_T\,(H^+)}{(H^+) + K_{a1}}\right]$$

$$\text{to get} \quad \text{rate} = \left[\frac{k_2(H^+)}{(H^+) + K_{a1}}\right](III)_T = k_{obs}\,(III)_T$$

Kinetic Derivation of k_3.- A kinetic treatment of equation k_3 assumes the existence of species $(III^=)$.

$$\text{Letting } (III)_T = (III^-) + (III^=)$$

$$\text{and, } \frac{Kw}{Ka_2} = K_b = \left[\frac{(III^-)(OH^-)}{(III^=)}\right]$$

$$\text{then } K_b = \left[\frac{(III^-)(OH^-)}{(III)_T - (III^-)}\right] \text{ and } (III^-) = \left[\frac{(III)_T\,K_b}{(OH^-) + K_b}\right].$$

$$\text{Assuming that rate} = \frac{-d(III)_T}{dt} = k_3\,(III^-)(OH^-)$$

$$\text{on substituting one gets rate} = \left[\frac{k_3(OH^-)\,Kb\,(III)_T}{(OH^-) + Kb}\right] = k_{obs}(III)_T$$

$$\text{or } \frac{1}{\text{rate}} = \left[\frac{(OH^-) + Kb}{k_3(OH^-)\ Kb\ (III)_T}\right] = \frac{1}{k_{obs}\ (III)_T}$$

Thus for $k_{obs} = \left[\frac{Kb\ k_3\ (OH^-)}{Kb + (OH^-)}\right]$ one can derive three special cases (Table I).

Total pH Profile.- The rate expression for the entire pH profile can be expressed by:

$$\text{Rate} = \frac{-d(III)_T}{dt} = \left[k_2\ (III) + k_3\ (III^-)\ (OH^-)\right].$$

If $(III)_T$ now $= (III) + (III^-) + (III^=)$ and

$$K_{a1} = \left[\frac{(III^-)\ (H^+)}{(III)}\right], \quad Kb = \left[\frac{(III^-)(OH^-)}{(III^=)}\right] = \frac{Kw}{K_{a2}}$$

and $Kw = (H^+)\ (OH^-)$ then by substituting and rearranging one can derive:

$$\text{rate} = k_{obs}\ (III)_T \text{ where}$$

$$k_{obs} = \left[\frac{k_2(H^+) + k_3\ K_1\ (OH^-)}{(H^+) + K_1 + \frac{K_1\ (OH^-)}{Kb}}\right]$$

$$\text{or } k_{obs} = \left[\frac{K_2\ (H^+) + \frac{k_3 K_1 Kw}{(H^+)}}{(H^+) + K_1 + \frac{K_1 Kw}{Kb(H^+)}}\right]$$

This reduces to the two simple cases when $(k_2 \rangle\rangle k_3)$ and $(k_3 \rangle\rangle k_2)$.

TABLE I. KINETIC RELATIONSHIPS FOR ALKALINE HYDROLYSIS OF ISOPROPYL 3-NITRO-o-HYDROXY-PHENYL METHYLPHOSPHONATE, (III).

	Condition	k_{obs}	$\frac{1}{k_{obs}}$
Overall		$\frac{Kb\ k_3(OH^-)}{Kb + (OH^-)}$	$\frac{1}{Kbk_3} + \frac{1}{k_3(OH^-)}$
Case 1	$Kb \gg (OH^-)$	$k_3\ (OH^-)$	$\frac{1}{k_3\ (OH^-)}$
Case 2	$Kb = (OH^-)$	$\frac{k_3(Kb)}{2}$	$\frac{2}{k_3Kb}$
Case 3	$Kb \ll (OH^-)$	k_3Kb	$\frac{1}{k_3Kb}$

EXPERIMENTAL

Materials

Isopropyl methyl phosphonofluoridate (Sarin),[22] (I), $CH_3(C_3H_7O)(PO)F$, purity of 98% or better based on P, F and isopropoxy group analysis, density of 1.08 g/ml at 25°, was prepared for this work by the Organic Branch, Chemical and Radiological Laboratories, Army Chemical Center, Md.

3-nitrocatechol, (II), was synthesized from catechol in the method described by Rosenblatt[23] or extracted from a crude mixture of nitrocatechol isomers available from Aldridge Chemical Co., Milwaukee, Wis. The extract was purified in the manner described[23] and 3-nitrocatechol was crystallized from 40-60° petroleum ether, (m.p. 86°). The compound exhibited at pH 1, λ_{max} 240, 298 mμ, ε 10,500, 12,500; pH 11, λ_{max} 303, 390 mμ, ε 6,700, 2,450; $\lambda_{isosbestic}$ 285, 314 mμ.

Isopropyl 3-nitro-o-hydroxy-phenyl methyl phosphonate, (III), $(C_3H_7O-)\left[(3\text{-}NO_2)(2\text{-}OH)C_6H_3O-\right](CH_3)PO$ was prepared by two methods. (A) Treatment of (I) with excess (II) in 100 ml of pH 6.5 phosphate buffer at 25° under an atmosphere of nitrogen yielded (III). After four hours, the reaction mixture was adjusted to pH 7.5 and extracted with 75 ml of ether. Upon evaporation, the ether residue was dissolved in 10 ml carbon tetrachloride and separated on a 30 gm

Celite column using 30 ml of pH 7.8 Borate buffer (0.1 M) as the internal phase. The column was eluted with carbon tetrachloride yielding (III) in the first fractions. Evaporation of the carbon tetrachloride yielded a yellow oil which decomposed at about 65° and yielded compound (IV) on exposure to air. The compound exhibited at pH 1, λ_{max} 278, 345 mμ, ε 7,800, 3,600; pH 11, λ_{max} 288, 415 mμ, ε 5,300, 6,300.

Anal. Calcd. for $C_{10}H_{14}O_6NP$: C, 43.6; H, 5.13; N, 5.1; P, 11.3. Found: C, 44.5; H, 5.2; N, 4.9; P, 11.4.

Continued elution of the column yielded a hydrolytic product of (III), 3-nitro-o-hydroxy-phenyl methyl phosphonate, (IV), $[(3\text{-}NO_2)(2\text{-}OH)C_6H_3O\text{-}](CH_3)PO(OH)$, which exhibited the following spectral properties: at pH 1, λ_{max} 212, 284, 350 mμ, ε 14,500, 6,600, 2,700; at pH 11, λ_{max} 232, 293, 426 mμ, ε 22,000, 6,600, 7,800. In diethyl ether, the compound exhibited λ_{max} 214, 282, 345 mμ, ε 7,800, 3,000, 1,300 and in carbon tetrachloride λ_{max} 280, 353 mμ, ε 7,500, 2,900. Continuous ethereal extraction of the above reaction mixture acidified to pH 1.0 permitted isolation of (IV). The compound was then crystallized from acetone and ether (m.p. 140.5-141.5°). Compound (IV) was soluble in water, methanol and ethanol, but slightly soluble in carbon tetrachloride and ether and insoluble in chloroform. Infra-red spectral analysis confirmed the assigned structure.

Anal. Calcd. for $C_7H_8O_6NP$: C, 36.06; H, 3.46; N, 6.01; Found: C, 36.25; H, 3.64; N, 6.32.

(B) An alternate method for synthesizing (III) in greater yield involved the reaction of dichloro-phosphine oxide, (VII), $(CH_3)PCl_2O$, (supplied by Org. Branch, Army Chem. Center, Md.) in equimolar quantity with (II), as shown in equation 7. The two compounds were allowed to react for two hours at 90°C in a round bottom flask. The reaction was accompanied by the evolution of hydrochloric acid gas and was considered complete when the gas evolution ceased. Treatment of the white crystalline solid product (VIII) with isopropanol yielded products (III) and (IV). These two resulting products were then chromatographically separated as described in method A above. UV and IR spectral determinations and elemental analysis confirmed that the two products (III) and (IV) were identical to those prepared from Sarin in method A.

Isopropyl methyl phosphonate, (V), $(C_3H_7O-)(CH_3)PO(OH)$ was provided by the Org. Branch, Army Chem. Center, Md., or prepared from the alkaline hydrolysis of Sarin[20] (sodium salt, m.p. 164-166°).

Methane phosphonic acid, (VI), $(CH_3)PO(OH)_2$ was provided by the Org. Branch, Army Chem. Center, Md. (white crystalline solid, m.p. 105°) (sodium salt, m.p. 221-223°C).

The isopropanol used was rendered essentially anhydrous (.05% H_2O) by treatment of reagent grade isopropanol as described by Vogel.[24] Barium hydroxide solutions were prepared in the manner described by Butler.[25] Corrections for appropriate ionic strength and temperature as affecting the partial dissociation of $Ba(OH)^+$ were made according to Gimblett and Monk[26] in order to determine the effective (OH^-) concentration of these solutions.

Nitrogen, as used in all kinetic runs, was passed over an Ascarite plug and bubbled through aqueous potassium hydroxide to remove any CO_2 present and prehumidified in a saturator at the reaction temperature. All solutions for kinetic studies and pK_a determinations were freshly boiled, CO_2 free, and were prepared with glass distilled water to minimize metal catalyzed reactions.

All other chemicals used in the preparation of buffers and kinetic solutions were analytical or reagent grade only. Compound (III) in anhydrous carbon tetrachloride and compound (I) were stored under refrigeration. Crystalline compounds (II), (IV), (V) and (VI) were stored in a vacuum desiccator.

Kinetic Procedures

A) Spectrophotometric Determination of k_1 and k_2.- The hydrolysis of isopropyl 3-nitro-o-hydroxy-phenyl methyl phosphonate,(III), was followed spectrophotometrically in the pH range 3-8 by determining the rate of appearance of (IV). Sarin and 3-nitrocatechol (mole ratio 1:5) were allowed to react in a solution of desired pH under a nitrogen atmosphere in a continuously stirred reaction vessel (fig. 1). The temperature was kept constant $\pm 0.05^{\circ}$ and the pH was maintained when necessary by the addition of base. Samples were removed periodically by pipette and rapidly transferred to a known volume of pH 3.0 phthalate buffer (0.05M). This solution was extracted exhaustively with ether to remove any excess 3-nitrocatechol and unreacted (III). The aqueous layer was made alkaline (pH 10) with sodium hydroxide and its absorbance was determined spectrophotometrically at 426 mμ. A modified procedure called for division of the reaction solution into three separate fractions after thirty minutes of reaction time had elapsed. Each fraction was then buffered at a constant desired pH value after the original solution had been run in a pH of 6.0. This enabled a substantial build-up of compound (III) in solution and eliminated a lag time before the appearance of (IV) could be observed (fig. 2). Values

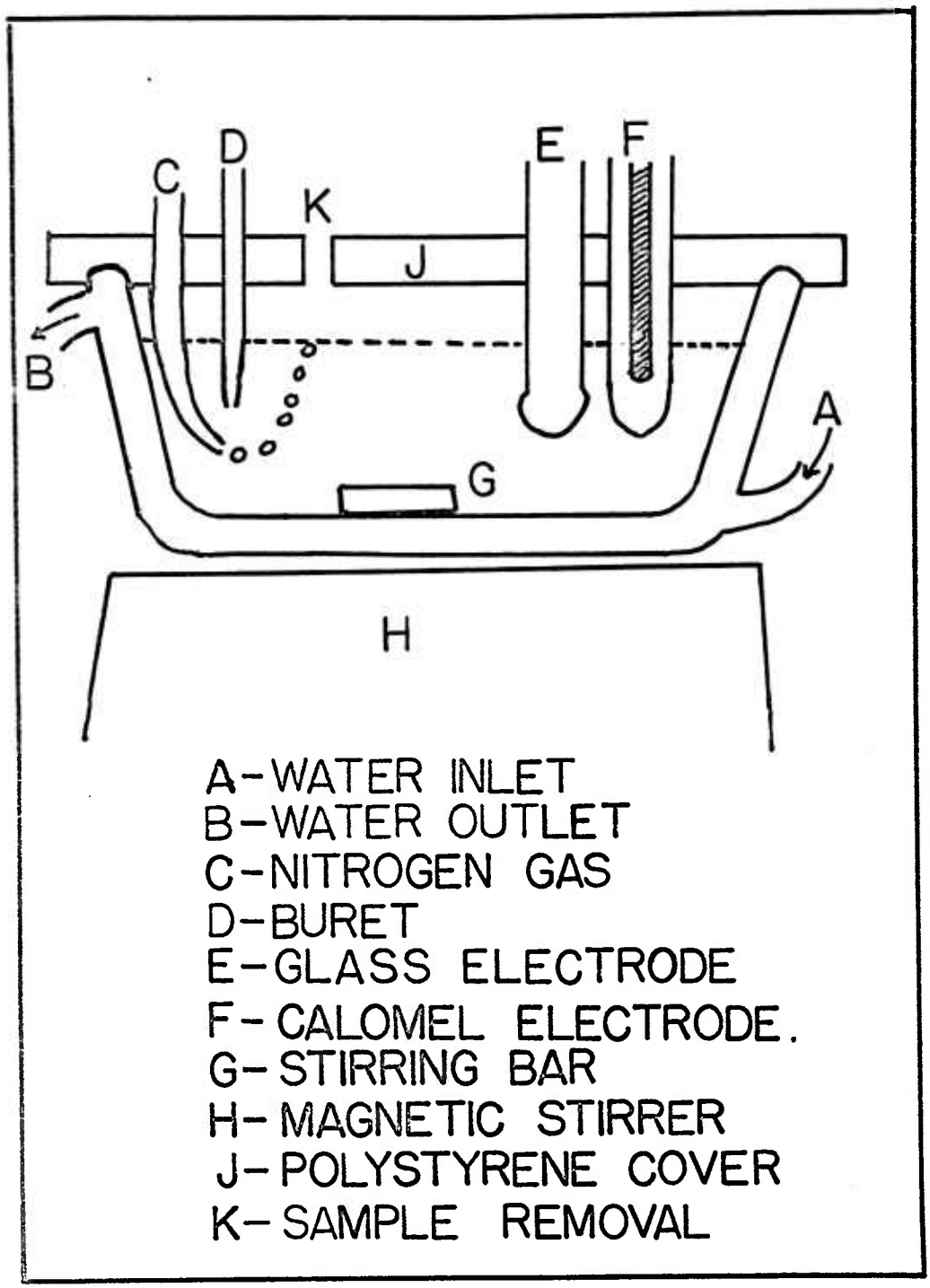


Fig. 1 REACTION ASSEMBLY

of k_{obs} determined in this manner were equal to those obtained in the original procedure, but allowed a more accurate determination of the rate at higher pH values. When reacion solutions were buffered to a desired constant pH level, several runs were repeated at the same pH, varying only the buffer concentration. Extrapolations were then made to zero buffer concentration when buffer catalysis was noted. The pseudo first order rate constants were calculated by Guggenheim's method[27] if the final reading was unknown.

B) Spectrophotometric Determination of k_s.- The hydrolysis of (III) under highly alkaline conditions (pH 10-13) was determined by following the rate of appearance of 3-nitrocatechol at its isosbestic point in the alkaline region. This wavelength was selected since 3-nitrocatechol oxidizes readily in this pH region and undergoes a spectral change depending on the extent of degradation. The absorbance reading at 314 mμ however, was insensitive to the extent of 3-nitrocatechol degraded and represented the total concentration of compound formed from the hydrolytic reaction. The absorbances were determined in the conventional manner in the Cary Model 15 Recording Spectrophotometer, thermostated to a temperature control of $\pm 0.1^{\circ}C$. The reactions were permitted to occur directly in the photometer cell. The barium hydroxide solutions were introduced by syringe into the cell

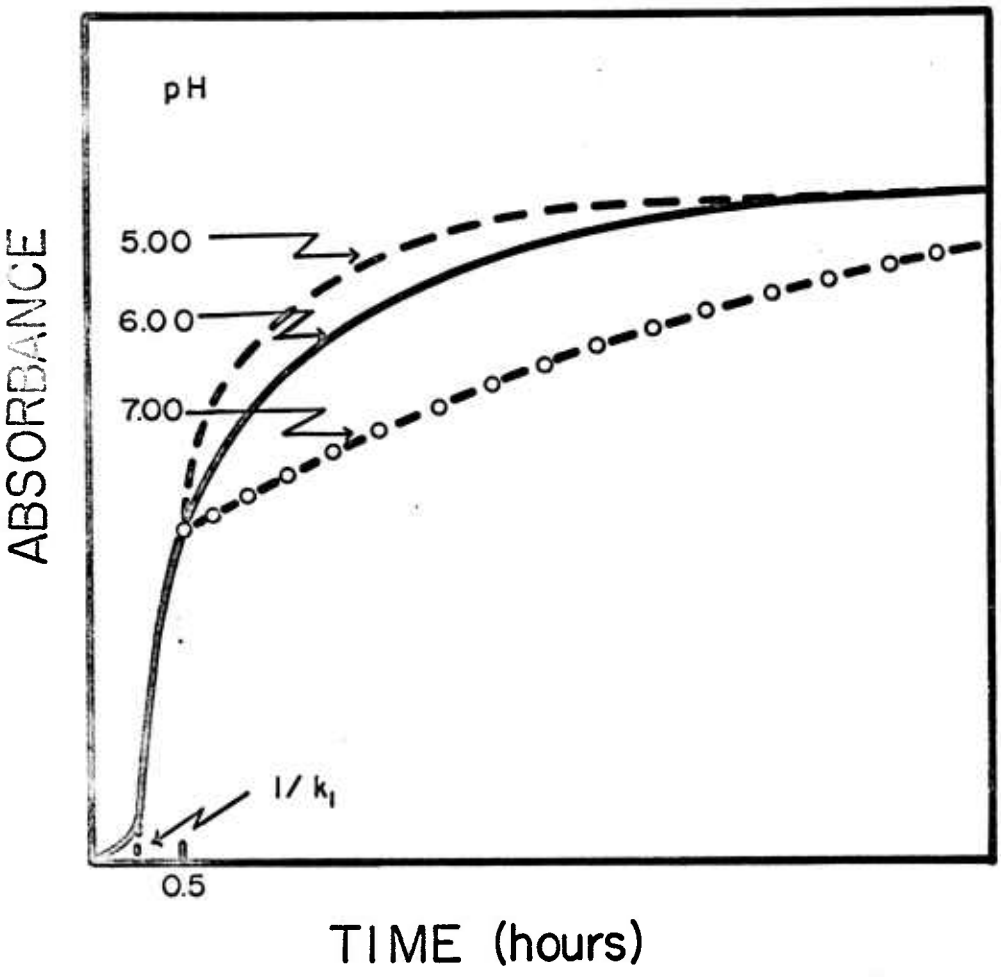


Fig. 2 Representation of method of determining rate of appearance of (IV) by separating reaction mixture after 0.5 hours into three separate fractions, each buffered accordingly. The plot shows absorbance reading of (IV) at 426 mμ vs time. The zero absorbance intercept, or lag time, is shown to be $1/k_1$.

which contained an isopropanol solution of (III). The total alcohol concentration was 2.5% and found to be negligible in affecting the rate of the reaction although it was used to determine the effective $(OH)^-$ concentration of each sample.

C) pH Stat Determination of k_2.- The pH stat assembly consisted of a Radiometer TTT1 Automatic Titrator with a SBR2 type Titrigraph, and a TTA3 Titration Assembly. The jacketted cell was maintained at constant temperature by circulation of water using a bath controlled to $\pm 0.05^{\circ}C$ with a Sargent Thermonitor. Samples employed were of 10.0 to 15.0 ml in volume and the volume of base (ca. 0.001 M NaOH) delivered from the microburet syringe did not exceed 0.50 ml during the course of any run. No effect in varying ionic strength was observed. The samples of (III) were prepared from fresh carbon tetrachloride solution. The carbon tetrachloride was flash evaporated using a Rinco flash evaporator and anhydrous isopropanol sample was replaced as the solvent. The isopropanol sample was then introduced into the reaction cell. The alcohol concentration did not exceed 5% in the actual experimental runs. The effect of isopropanol concentration on the rates was determined and values were extrapolated to zero alcohol concentration.

Analytical Procedures

Chromatographic separation of the components of the reaction mixture at various pH values was effected by using both thin layer chromatography (TLC), and descending paper chromatography. TLC sample loads of 10-100γ were applied to plates prepared from Silica Gel G or Silica Gel GF_{254}[28] in the conventional manner.[29] In some identifications the adsorbent was treated with a buffer solution before activation by heating. Developing solvents used were methanol: pyridine (4:1) and methanol: chloroform (1:3). Compounds (II), (III) and (IV) were readily identified by the aromatic nitro chromophore, visible also as a strong fluorescence in the short wave UV light. The phosphonic acids (V) and (VI) were detected by direct analysis of P using the method of Boltz and Mellon[30] as modified by Crowther.[31] An acid molybdate spray was used to form a complex which upon heating and exposure to UV light reduced to a blue color.

Whatman No. 1 and No. 4 chromatographic grade paper were used for descending techniques. Development was carried out at 30°C using two solvent systems, neutral: butanol; ethanol; water (2:1:1) and basic: butanol; butyl acetate; pyridine; water (30:15:10:50)(organic layer). In all cases, the development of spots included simultaneous parallel development of known compounds from the reaction scheme, previously

isolated. The paper chromatographic technique was preferred for quantitative P determinations since the assay[32] for total phosphorus used, utilized total digestion of the chromatogram. Primary potassium orthophosphate, KH_2PO_4 was used as a reference for standard calibration curves.

Determination of Dissociation Constants

The acid dissociation constants of (III), K_{a1} and (IV), K_{a2} were determined spectrophotometrically using the method of Flexer et al.[33] The value of K_{a2} for (IV) was also determined by potentiometric titration.

The K_{a1} value of (IV) was determined by partition coefficient whereby solutions of the compound in varied hydrochloric acid concentrations were extracted with ether and the absorbance determined spectrophotometrically at 282 mμ.

The second dissociation constant ($K_{a2}=\frac{Kw}{Kb}$) of (III) was determined kinetically from the hydrolytic data at high pH. Dissociation constants for (V) and (VI) were determined by potentiometric titration using the Radiometer Titrigraph apparatus.

RESULTS

Establishment of Reaction Products.- The initial product isolated from the reaction of Sarin and 3-nitrocatechol was (III). This ester was separated chromatographically from the reactants and characterized by UV, IR and elemental analysis. A comparison of this product with that synthesized in reaction 8 from 3-nitro-o-phenylene methyl phosphonate,(VIII), (cf. supra) showed that the two esters were identical and corresponded to the meta-linked isomer of isopropyl 3-nitro-o-hydroxy-phenyl methyl phosphonate (III). Thus compound (III'), if formed, was either quickly transformed to its isomer, (III), or was present in too small a concentration to be detected and isolated in the chromatographic separation.

The hydrolytic products of (III) obtained over a pH range 1.0 to 13.0 were characterized. The ester was placed in various buffered solutions at 30.0° and samples were taken at 4 hours, 24 hours, 7 days and 21 day periods for separation by TLC. A composite chromatogram showing the location of spots identified after 21 days of reaction is shown in figure 3. Known compounds were run simultaneously with each reaction mixture to aid identification. Compounds (II), (III) and (IV) were identified by detection of the nitro group. Compound (IV), 3-nitro-o-hydroxy-phenyl methyl phosphonate,

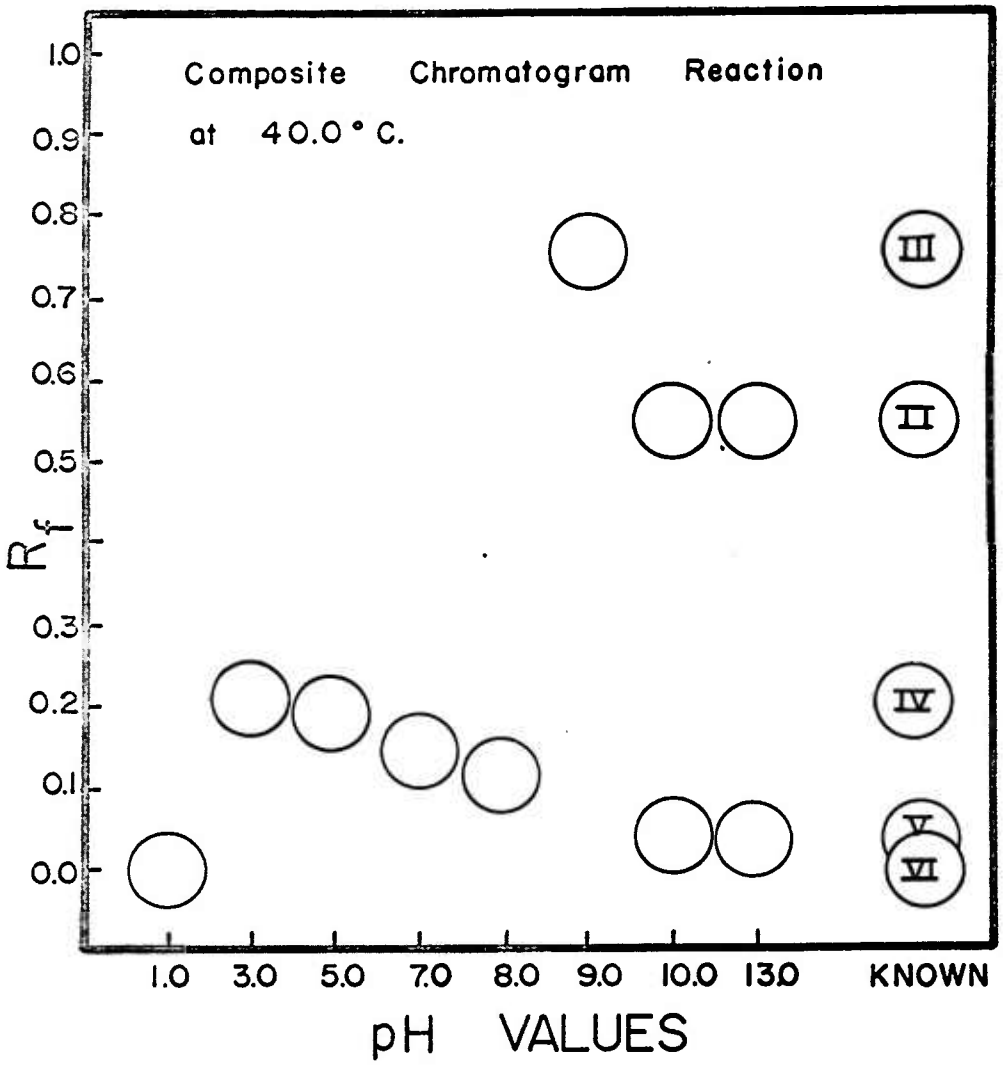


Fig. 3 Composite chromatogram showing locations of spots which represent products isolated from the hydrolysis reaction of (III) at various pH values. Parallel runs of known compounds and their R_f values are shown at the right.

showed a buffer effect when developed in the pH 3-8 region due to partial dissociation. The phosphonic acids (V) and (VI) were identified by P assay. It is apparent from the diagram that the hydrolytic pathway changes at about pH 9.0.

A) Spectrophotometric Determinations of k_1 and k_2

Determination of Reaction Constants.- The basis for the spectrophotometric determinations of the rate of appearance of (IV) was the reaction of Sarin with 3-nitrocatechol (eq. 1) followed by the hydrolysis of the intermediate ester to the desired product (eq. 2). The value of k_1 for this reaction has been previously determined by Epstein et al[17], who report a value of 1.5(1 $mole^{-1}$ min^{-1}) as the apparent second order rate constant at 25°C.

Since the absorbance readings were directly proportional to the concentration of (IV), the rate constants could be calculated directly from a plot of time vs absorbance of (IV) after removal of other reactants as shown in figure 2 for pH 6.0. The slope of the linear portion of the plot was equal to k_2' and the intercept of this line was $1/k_1$. The modified procedure as shown for pH values of 5.0 and 7.0 did not permit an evaluation of k_1. Values of k_1' as determined from k_1 and extrapolated to 25° were in good agreement with the values obtained by Epstein[17]. Actual values of k_2' however, were determined from semi log plots of absorbance vs time. When

at higher pH values, A_∞ could not be determined by conventional means, the Guggenheim method was used. Excellent agreement between both methods was observed at pH values where both determinations were made.

Dependence of the Reaction Rate on pH.- The pH of the reaction was kept constant by using various buffers. The ionic strength was kept constant at 0.6 by the addition of sodium chloride. Since these hydrolytic reactions exhibited general base catalysis as shown by Russo[18] and others, the effect of varying buffer concentrations was studied and the results are shown in figure 4. The value of k_2 was determined by extrapolating k_2' to zero buffer concentration and dividing by the initial Sarin concentration. The pH dependency of the reaction in the pH range of 3.0 to 8.0 at 40° is shown in the lower curve of figure 5. The theoretical curve is drawn from a value of k_2 as determined by pH stat methods (cf. infra) at pH 5.0.

B) Spectrophotometric Determination of k_3

Order of Reaction and pH Dependency.- The rate of alkaline hydrolysis of (III) was observed on the isolated ester, the hydrolysis being followed by the rate of appearance of the 3-nitrocatechol moiety spectrophotometrically at its isosbestic point. A plot of log k_{obs} vs pH for this system is shown in figure 6. Corrections were made for the incomplete

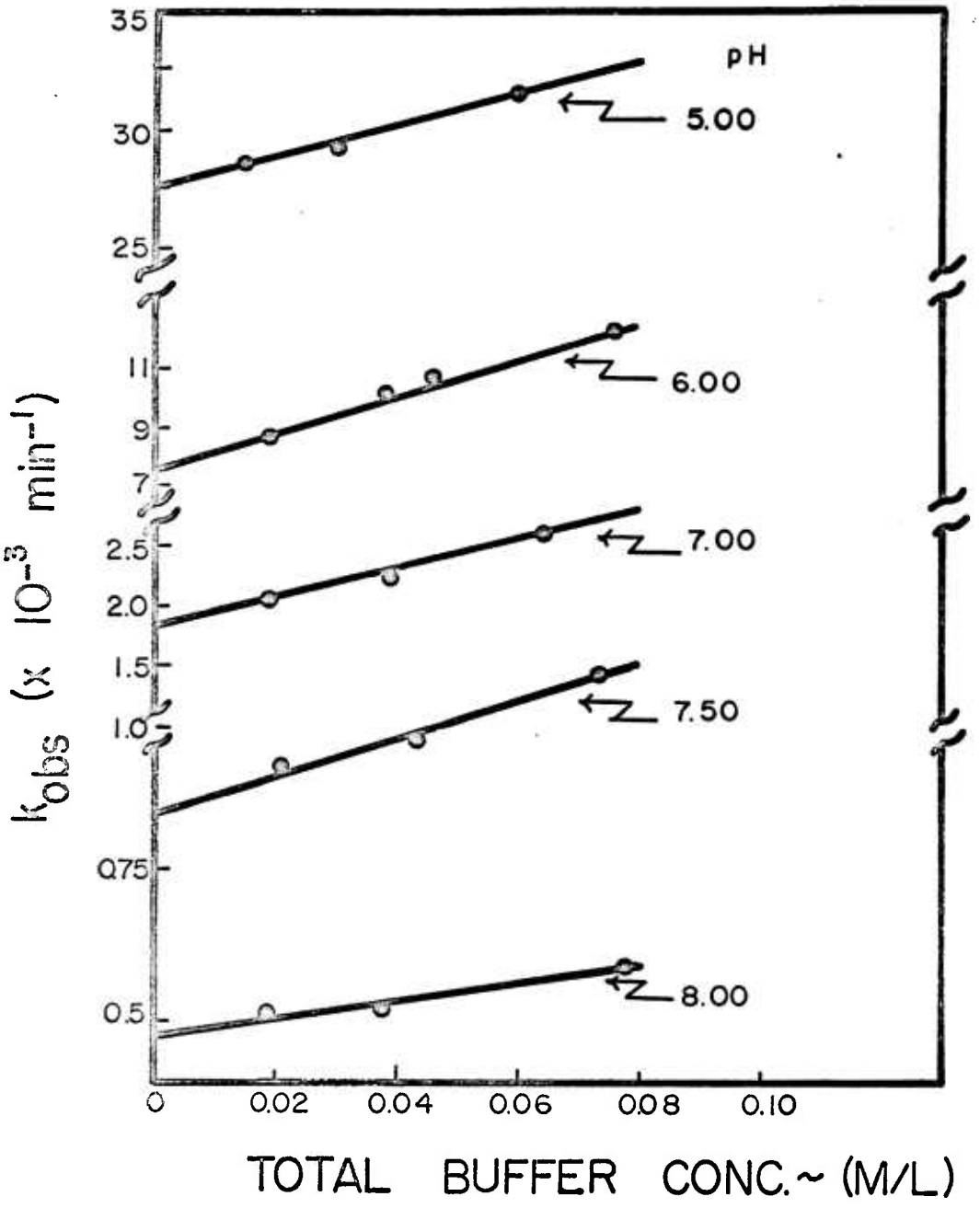


Fig. 4 Effect of changing buffer concentrations on the observed reaction rate at constant pH and 40.0°C.

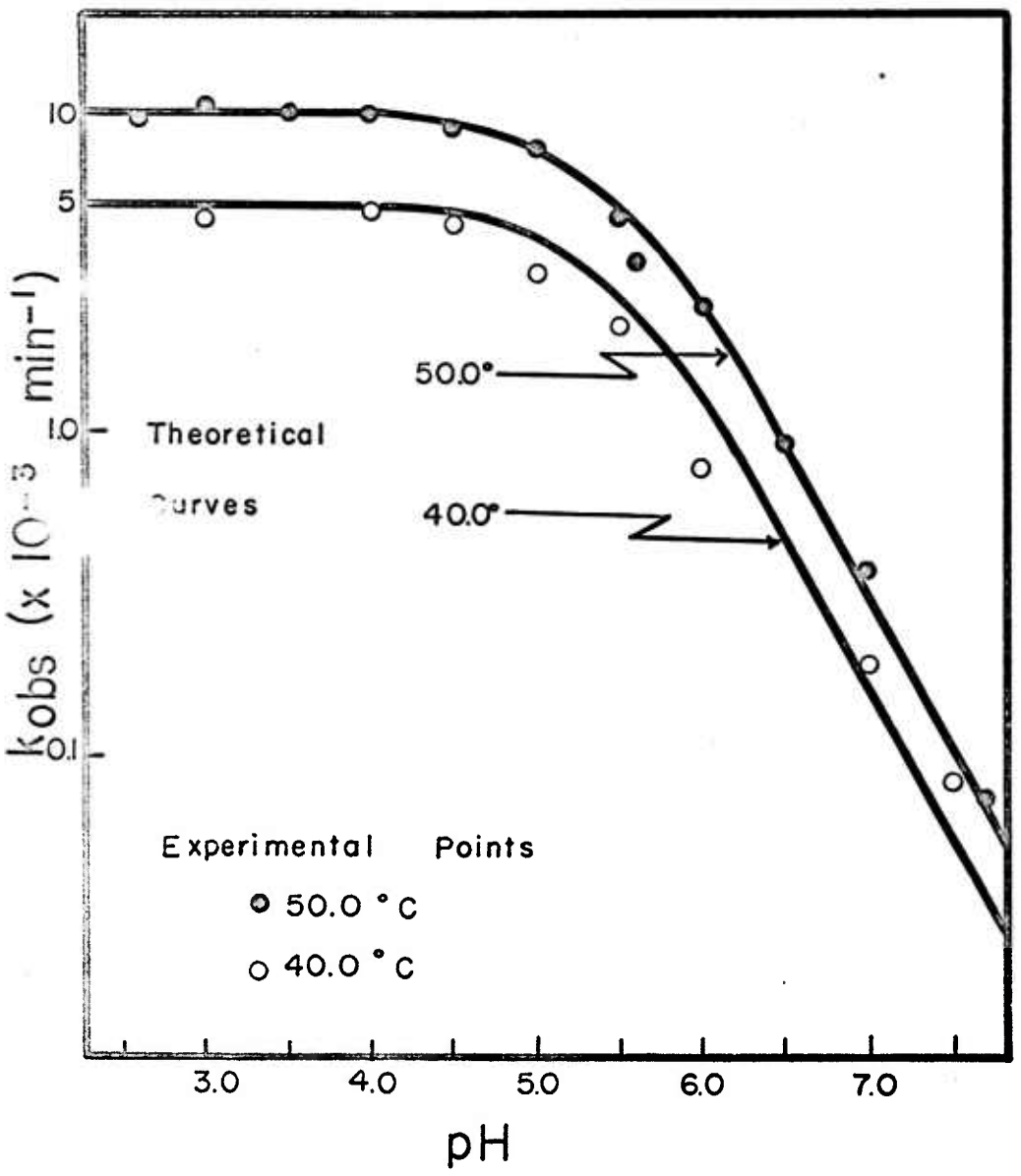


Fig. 5 The pH - rate profile for reaction k_2 at 40.0 and 50.0°C where the circles are actual experimental points and the solid lines correspond to the theoretically expected results.

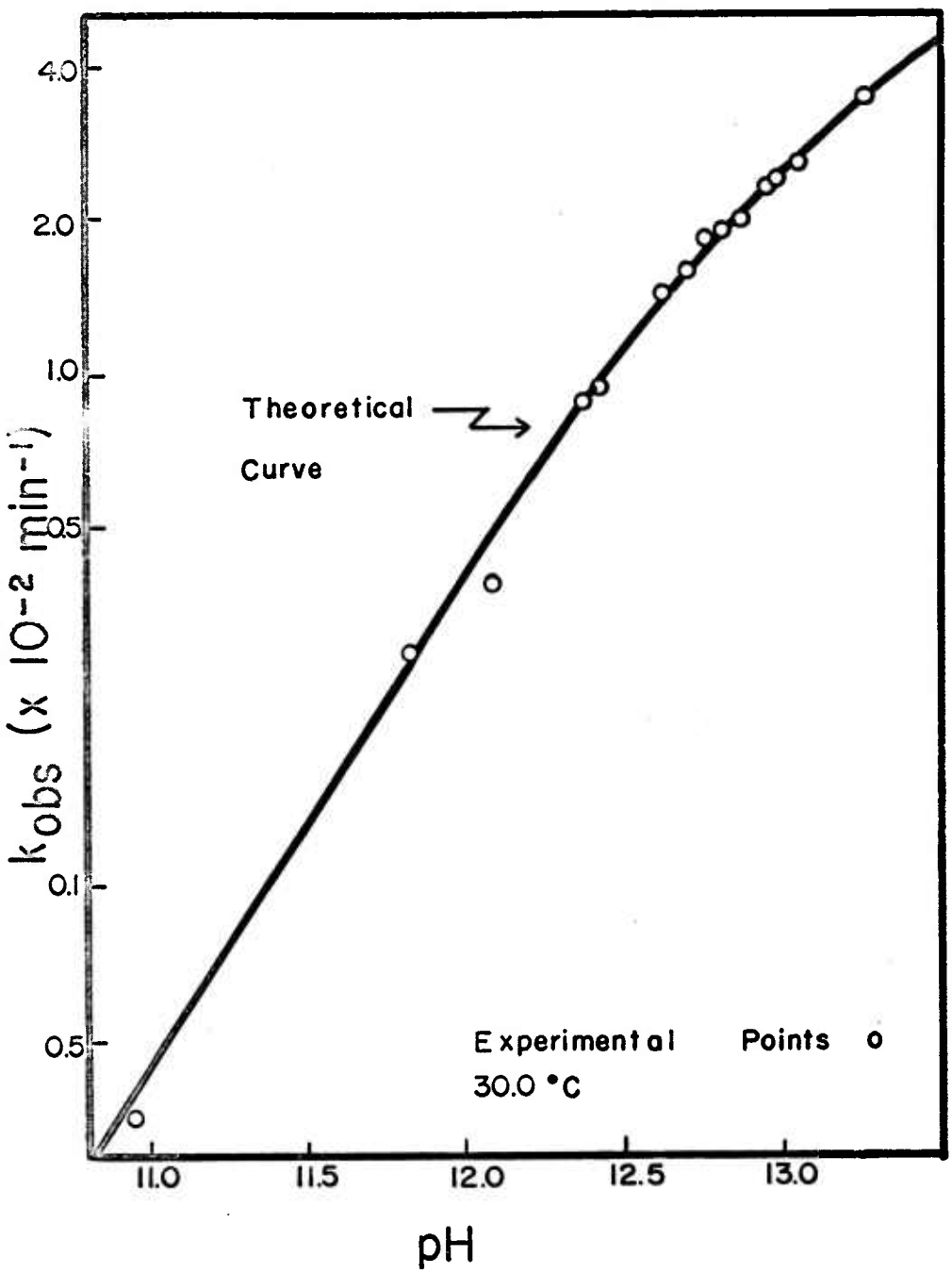


Fig. 6 The pH - rate profile for reaction k_3 at 30.0°C where the circles represent experimental results and the solid curve is the calculated theoretical value.

dissociation of $Ba(OH)^+$. The total % dissociation of barium hydroxide was determined from Gimblett's data by plotting μ vs log dissociation. (The Huckel equation used would be of the form $pOH = \frac{Az^2\sqrt{\mu}}{1+a_iB\sqrt{\mu}} - C\mu$ where $C\mu$ is the correction term for $\mu \gg 0.1$.) A straight line was obtained from which the dissociation of $Ba(OH)^+$ was calculated and the effective $(OH)^-$ determined for each ionic strength at 30.0°C. A reciprocal plot (fig. 7) showing $1/k_{obs}$ vs $1/(OH)^-$ gave an essentially linear relationship based on the kinetic treatment of equation 3 which assumed the existence of species (III=). The slope of the line in figure 7 was calculated as 3.5 and the intercept, 16 min. from which k_s and Kb were calculated. At the point where $\frac{1}{Kb} = \frac{1}{OH}$ (Case II) the value of $\frac{1}{k_{obs}}$ should equal twice the intercept ($\frac{1}{KbK_s}$). The value of 32 min. verifies this internal check.

C) pH Stat Determinations of k_2.- The results of the hydrlytic reaction of (III) measured by pH stat indicated that the reaction proceeds at rates directly proportional to the concentration of undissociated species (III). Plots of the logarithm of the residual ester (III) versus reaction time yielded essentially straight lines. Actual plots of log $(V_\infty - V_t)$ versus time related the volume of titrant used to the amount of (III) hydrolyzed. Since each sample was introduced in isopropanol, the half-life calculated for each

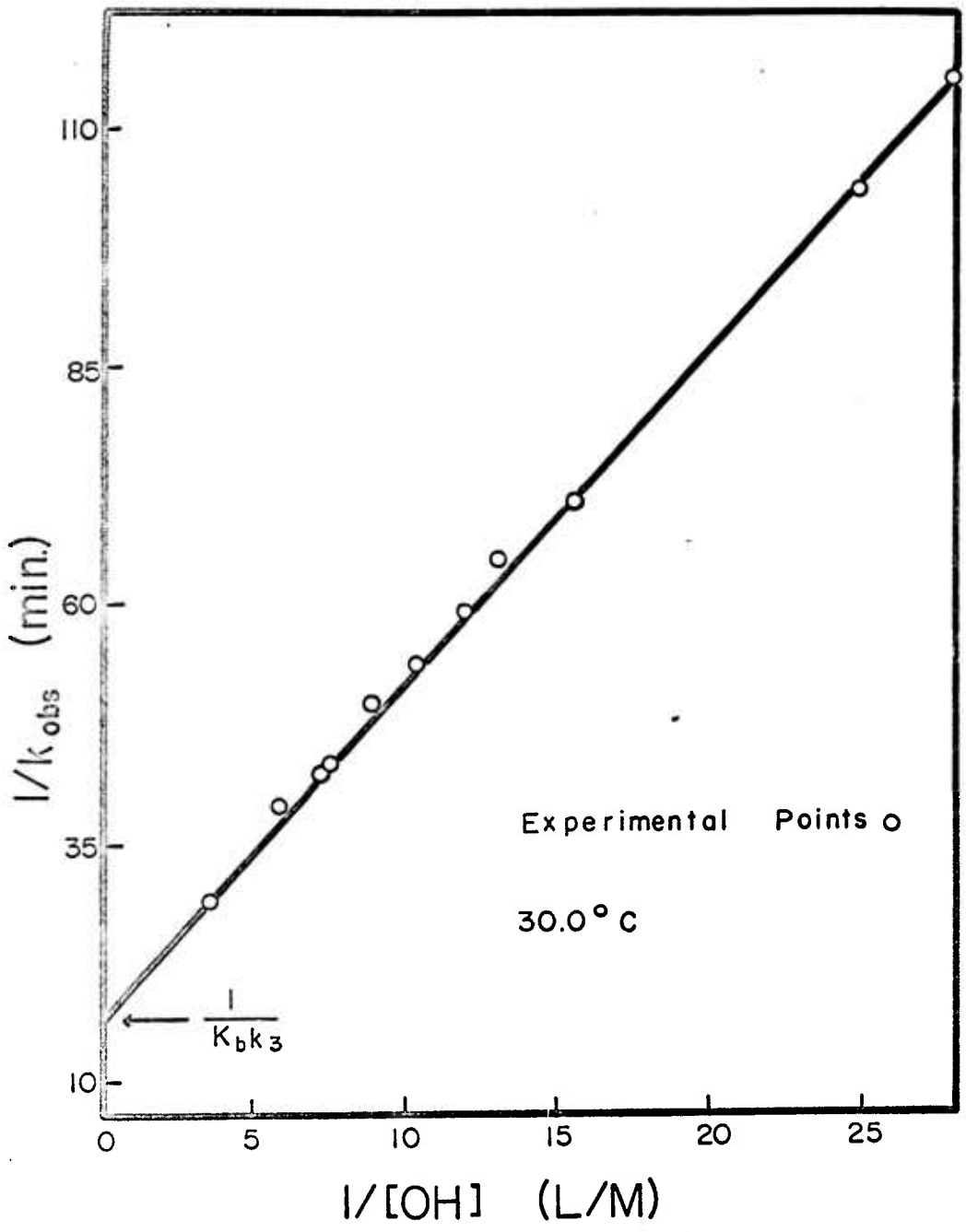


Fig. 7 Plot of reciprocal observed reaction rate vs reciprocal $(OH)^-$ concentration showing essentially linear relationship with intercept value of $\frac{1}{K_b k_3}$.

reaction was corrected to zero alcohol concentration as shown in figure 8. The value of K_{a1} was determined spectrophotometrically and is shown in Table II.

TABLE II. DETERMINATIONS OF ACID DISSOCIATION CONSTANTS FOR ALL REACTION SPECIES INVOLVED IN ISOPROPYL 3-NITRO-o-HYDROXY-PHENYL METHYL PHOSPHONATE HYDROLYSIS.

Compound	pK_{a1}	pK_{a2}
3-nitrocatechol II	6.66	
Isopropyl 3-nitro-o-hydroxy-phenyl methyl phosphonate III	5.53	13.49 *
3-nitro-o-hydroxy-phenyl methyl phosphonate IV	1.15	6.78
Isopropyl methyl phosphonate V	2.55	
Methane phosphonic acid VI	2.30	7.91

(*Determined from $pK_{a2} = pKw - pKb$ at 30.0°C)

The calculated first order rate constants were found to be identical within experimental limitations to those determined from the theoretical curve. The half-life values were constant under varying initial concentrations of (III). The pH dependency of the reaction is shown in the plot of log k_{obs} vs pH in figure 5 for 50.0°(upper curve) and figure 9 for 30.0°(acid region). Attempts to measure the rate of hydrolysis at higher pH values were unsuccessful due to the slowness

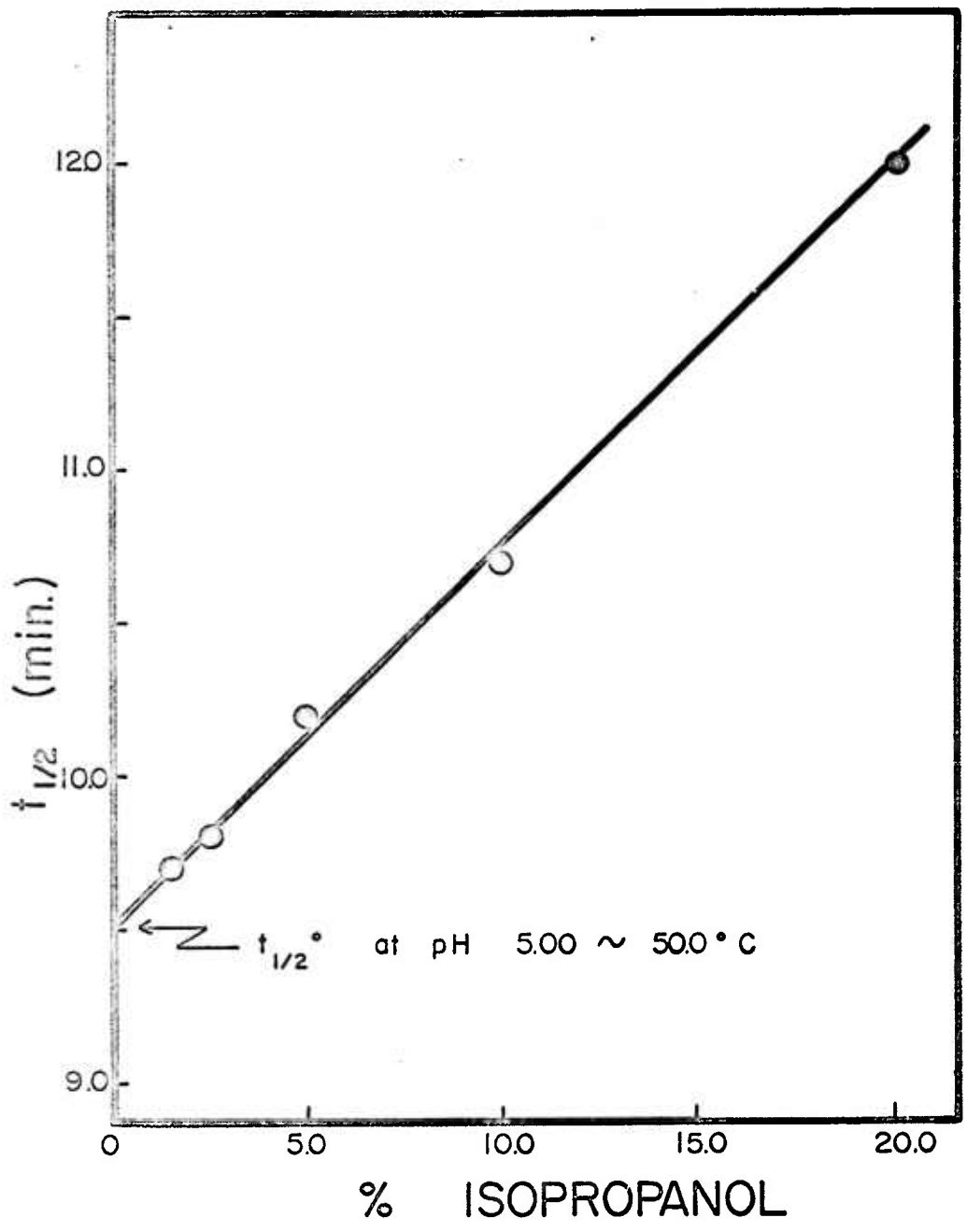


Fig. 8 Effect of varying isopropanol concentration on half-life of reaction k_2 at constant pH = 5.00 and 50.0°C.

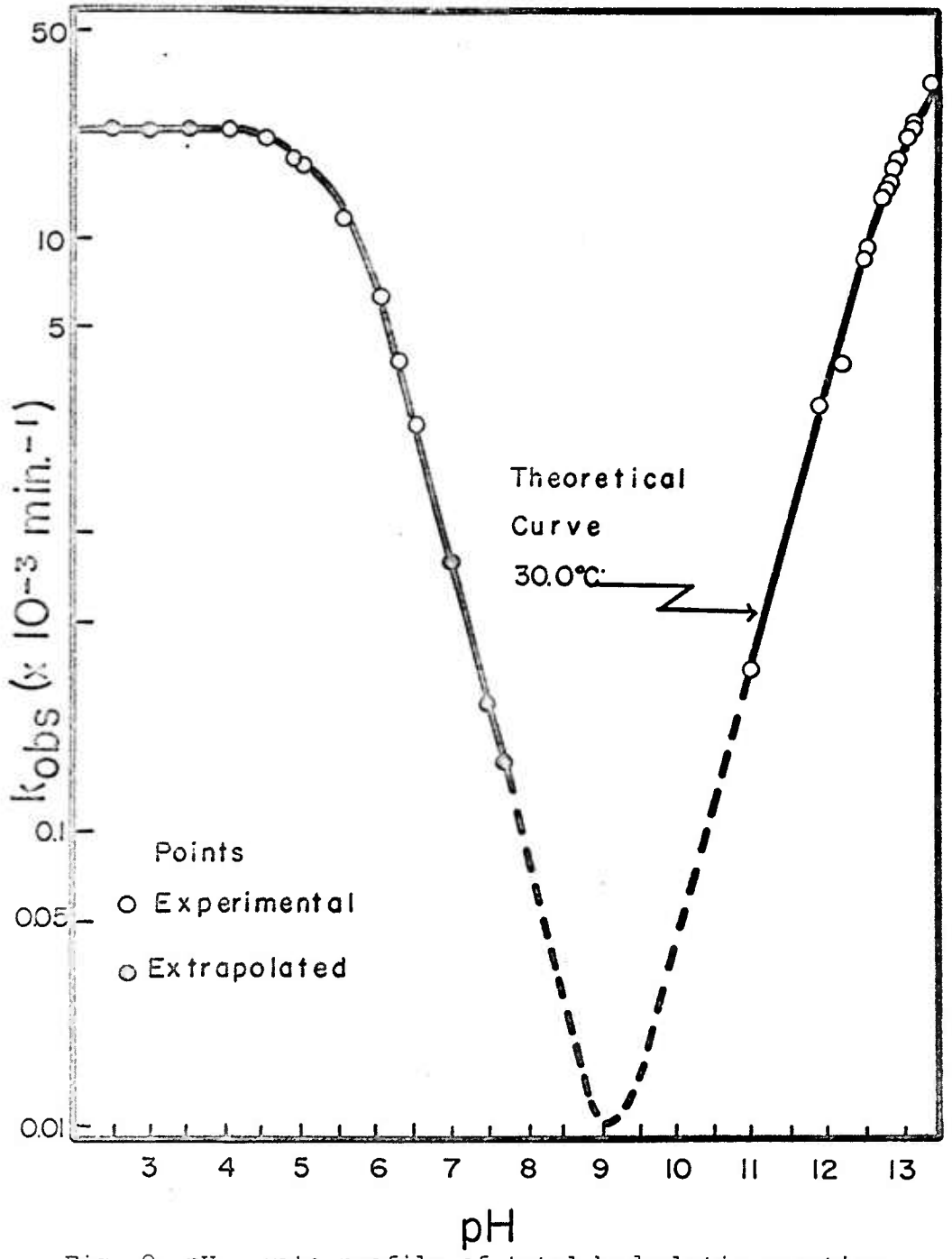


Fig. 9 pH - rate profile of total hydrolytic reaction (k_2+k_3): solid line showing calculated theoretical curve; broken line showing projected theoretical curve; and circles showing experimental values determined at 30.0 or extrapolated from 50.0°C.

of the reaction and CO_2 interference.

D) Total pH Profile.- The overall pH profile at 30.0° is shown in figure 9. The solid circles represent experimental points extrapolated from 50.0°C. The slope of the curve from pH 6-9 is essentially -1.0 and that from pH 9-12 is 1.0.

The behavior of 3-nitro-o-hydroxy-phenyl methyl phosphonate, (IV), was studied separately at pH 1.0 to 13.0 at 30.0° and 90.0°C. No visible evidence of any degradation could be seen after one week at any pH except pH 1.0 (pH 3.0 determinations showed no degradation). Methane phosphonic acid and 3-nitrocatechol were detected as hydrolytic products of the degradation in 0.1 N hydrochloric acid.

Determination of the Apparent Activation Energies of the Hydrolytic Reactions.- The apparent activation energies of the reaction were determined by measurements of the observed rate constants at varying temperatures according to the Arrhenius equation: $\frac{d(\ln k)}{d(1/T)} = \frac{-Ea}{R}$. The determination of the temperature dependency of k_2 was performed at pH 5.00 by pH stat methods and is shown in figure 10. The value obtained at 40.0° was used as a basis for calculating the lower theoretical curve of figure 5. The temperature dependency of k_3 was determined spectrophotometrically at pOH = 1.01 (fig. 11). The $(OH)^-$ was maintained constant since the pH dependency at varying temperatures would be

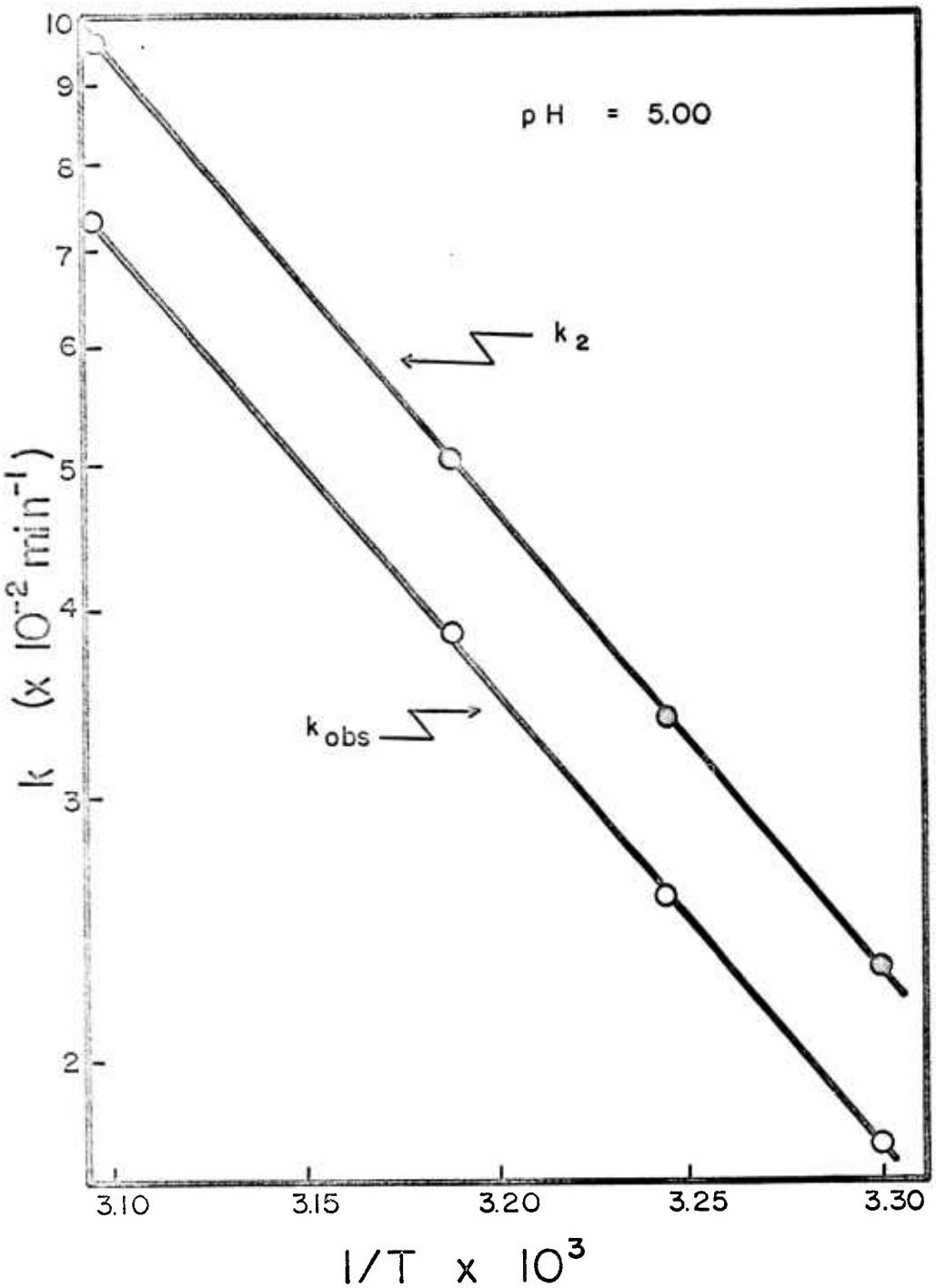


Fig. 10 Arrhenius type plot of logarithm of observed rate constant and k_2 vs reciprocal absolute temperature at constant pH = 5.00.

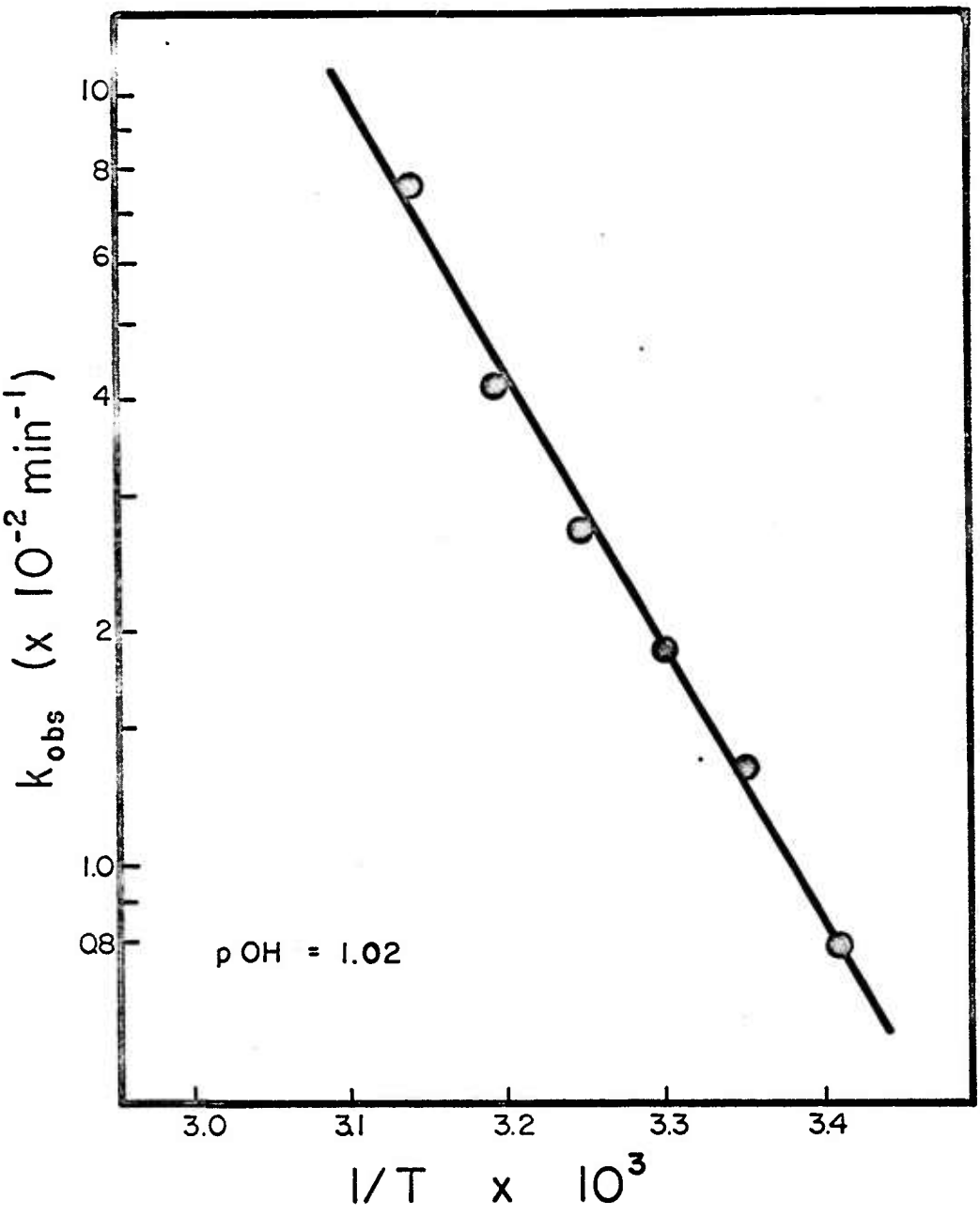


Fig. 11 Arrhenius type plot of logarithm of observed rate constant vs reciprocal absolute temperature for the hydrolysis of (III) at constant pOH = 1.02.

dependent on Kw. Values for the respective rate constants and activation energies are given in Tables III and IV.

TABLE III. RATE CONSTANTS DETERMINED AT VARIOUS TEMPERATURES.

	30.0°	40.0°	50.0°
$k_2(x10^{-2})(min^{-1})$	2.3	5.1	9.6
$k_3(x10^{-1})(l\ mole^{-1}min^{-1})$	2.8_6	6.3	15.4
$Kw(x10^{-14})$	1.4_7	2.9_2	5.4_7

TABLE IV. APPARENT ACTIVATION ENERGIES FOR HYDROLYSIS SCHEME OF ISOPROPYL 3-NITRO-4-HYDROXY-PHENYL METHYL PHOSPHONATE.

Reaction		$E_a(Kcal.\ mole^{-1})$
eq. 3 (k_2, k_{obs})	pH = 5.00	13.9
eq. 4 (k_{obs})	pOH = 1.01	14.3

DISCUSSION

Hydrolysis of Isopropyl 3-nitro-o-hydroxy-phenyl Methyl Phosphonate (III)

The Similarity of Reaction Profile to "Ageing".- The total pH profile for the hydrolysis of (III) as shown in figure 9 resembles that of the "ageing" phenomenon of inhibited cholinesterases. The hydrolysis is shown to be acid catalyzed in the physiological pH region 6-8. Furthermore, from the products isolated, one may conclude that the reaction responsible for this catalysis is the dealkylation of the isopropyl moiety by intramolecular facilitation of the vicinal hydroxide of the 3-nitrocatechol substituent. Only at extremely high pH (10-13), is this effect reversed so as to provide cleavage of the aromatic moiety which would be the expected leaving group in the absence of any neighboring group influence.

In addition, "ageing" in previously reported[11] inhibited cholinesterase reactions was accompanied by the isolation of isopropanol similar to the cleavage of the isopropyl moiety in the 3-nitrocatechol model presented here. Previous reaction profiles of Sarin phenolate and catecholate esters have shown no evidence of significant acid catalysis, although dealkylation was observed in the case of catechols. The present study demonstrates that a model selected, a priori,

because of the facilitative role of the ortho hydroxy group and the stereoelectronic properties of the molecule, can be shown to behave in a manner similar to that of the freshly inhibited enzyme when "ageing".

Hydrolysis of (III) - Dealkylation(k_2).- The hydrolysis of Sarin is catalyzed by various nucleophilic species, but the reaction of catechols with Sarin fails to be truly catalytic because the attacking nucleophile is not regenerated. Thus, in the hydrolysis of (III), instead of the normally less nucleophilic 3-nitrocatechol moiety being cleaved, the isopropyl group is hydrolyzed yielding a very stable ester, (IV), which was isolated and identified. The effective nucleophilic agent in this hydrolytic reaction is probably water. The fact that the monoanion of isopropyl 3-nitro-o-hydroxy-phenyl methyl phosphonate (III^-) is relatively resistant to hydrolysis as compared to the neutral species (III), suggests that the oxygen of the water dipole is repelled by the negative charge of the ionized ester.

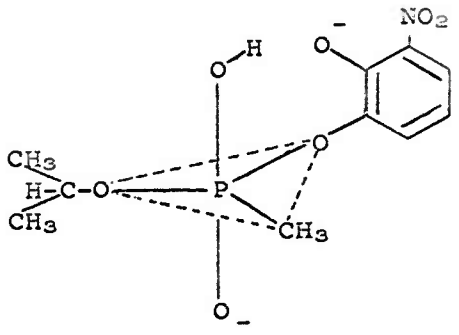
The cleavage of the isopropyl moiety is preferred because of the intramolecular facilitation of the o-hydroxy group. The effect of this group may be due to a) anchimeric assistance in the removal(solvolysis) of the neighboring isopropyl group, or b) the stabilizing effect of hydrogen bonding between

the phosphonyl oxygen and the vicinal hydroxide. In either case the neutral species hydrolyzes exclusively by isopropyl cleavage.[34] This is analogous to the "ageing" reaction in which a similar dealkylation yields the same product.

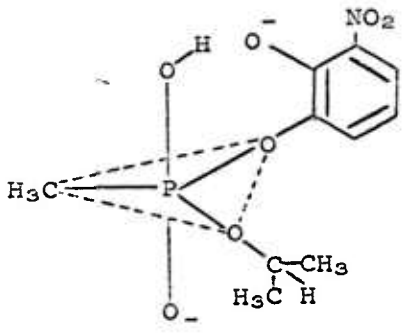
It is noteworthy that the hydrolytic product of the k_2 reaction which was isolated, 3-nitro-o-hydroxy-phenyl methyl phosphonate, (IV), is extremely resistant to further hydrolysis in the anion or dianion form. Likewise, the "aged" enzyme in cholinesterase studies is also resistant to such reactivation, (a dephosphorylation process), and it is this resistance which is the basis for the irreversible nature of the enzyme inhibition. The neutral species of (IV), however, does undergo hydrolytic reaction (eq. 5) in a manner somewhat analogous to the behavior of diester phosphates.[35] Furthermore, the effects of this reaction were observed in attempts to study the hydrolysis of (III) at pH values below 2.50 by pH stat methods.

Aromatic Cleavage in Alkaline Hydrolysis (k_3).- Under extremely alkaline conditions, (III) undergoes a hydrolytic elimination, (as shown in eq. 4), of the 3-nitrocatechol which is identified in the reaction products. Under these circumstances the stability of the P-O-C (aromatic) bond is weakened in the dianion and the catecholate dianion is the

favored leaving group. A pentacovalent dianion ($III^{=}$) may be postulated in the probable mechanism for this reaction. Recent work on the displacement reactions of optically active phosphonium compounds,[36] and the alkaline decomposition of such compounds[37] is based on the formation of such a pentacovalent intermediate. During formation of the pentacovalent intermediate, it seems most reasonable to expect a back side attack by hydroxide ion to form the conjugate base which would give a trigonal bipyramidal structure as shown in (IX_a) and (IX_b).



(IX a)



(IXb)

The ease of hydrolysis of the pentacovalent intermediate can by correlated with the stability of the displaced anion, as judged by the second dissociation constant of 3-nitrocatechol. It should be noted that the availability of the d orbitals of phosphorus for bonding during the formation of the pentacovalent intermediate is markedly affected by the phosphorus substituents. This effect coupled with the participation of the vicinal hydroxide are the principal contributors to the stereoelectronic effects which are responsible for the unusual mode of hydrolytic cleavage of isopropyl 3-nitro-o-hydroxy-phenyl methyl phosphonate.

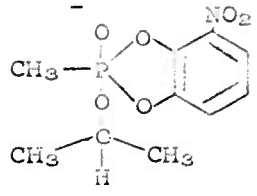
It is quite probable that the k_3 hydrolytic reaction may be catalyzed by the presence of barium ion. Such catalysis of phosphate diesters is well known[38] and would favor P-O cleavage over that of C-O.

Formation of Isopropyl 3-nitro-o-hydroxy-phenyl methyl phosphonate, (III) (k_1)

Role of Neighboring Hydroxyl Groups.- One might expect that since the attacking nucleophile in the reaction of Sarin and 3-nitrocatechol (k_1) is the ortho monoanion, of the two possible position isomers of isopropyl 3-nitro-o-hydroxy-phenyl methyl phosphonate, the ortho linked ester, (III'), would be formed first and predominate as the principal reaction product (eq. 1). However, the isolation of only the

meta-linked ester, (III), as the reaction product indicates that this isomer is favored thermodynamically and is probably formed by phosphoryl migration involving the vicinal hydroxyl group. A mechanism which is consistent with these results can be postulated showing a cyclic intermediate (X). The formation of (III) from the cyclic ester, 3-nitro-o-phenylene methyl phosphonate (VIII) in equation 8, demonstrates that such a pathway is indeed probable.

The rapid reaction of catechols with Sarin (as compared with phenols) has been attributed to participation of the undissociated o-hydroxy group. The anchimeric assistance that this group gives to both the nucleophilic attack and phosphoryl migration in k_1 is also the basis for the unusual hydrolytic behavior of (III).



(X)

Conclusion

The agreement of the theoretical curve with the observed experimental results in figure 9 demonstrates that a mechanism involving the reaction of the neutral species of isopropyl 3-nitro-o-hydroxy-phenyl methyl phosphonate, (III) and the dianion, ($III^{=}$) is consistent with the results shown. The possibility, however, of other kinetically equivalent mechanisms representing the true reaction is acknowledged. However, the probability that reaction k_2 is between a conjugate acid of (III) and a hydroxide ion is remote as cited by Vernon in his discussion on phosphate diesters.[35] It is generally known also, that aromatic phosphoric ester hydrolysis[39] is neither hydrogen ion nor hydroxyl ion catalyzed over the pH region (1-10).

The pH dependency shown in "ageing" curves reported in previous studies[12] is characterized by a profile which is closely parallel to that shown in figure 9. The role of intramolecular catalysis most probably is responsible for the mechanistic behavior of the Sarin-nitrocatecholate ester system. This intramolecular catalysis may be likened to the enzymatic catalysis in that the substrate is constrained in a position near the catalytic group. The demonstration, that the model of the Sarin-3-nitrocatecholate ester reported here undergoes an intramolecularly facilitated dealkylation,

provides an insight into the possible molecular mechanism involved in the essentially irreversible inhibition of "aged" enzymes. In seeking answers to the mechanisms of action of these organophosphorus poisons, investigations can yield invaluable tools for the study of normal cell physiology.

SUMMARY

The present investigation was designed to determine whether the "ageing" reaction observed for cholinesterase inhibited by phosphorylating agents could be simulated by a model system composed of catalytic species and a known inhibitor. On the basis of its structure, 3-nitrocatechol was selected as the simulant for the enzyme in its reaction with Sarin (isopropyl methyl phosphonofluoridate). The kinetics and mechanism of the hydrolytic cleavage of the resulting intermediate ester product, isopropyl 3-nitro-o-hydroxy-phenyl methyl phosphonate, were investigated in the pH range 2.5-13.5. The mechanism of hydrolysis, as determined kinetically and by product isolation, indicated that the major reaction, primarily responsible for the hydrolytic cleavage in the pH range 2.5-8.0, closely resembled the process apparently responsible for the irreversibility gradually produced in the freshly inhibited enzymes.

The hydrolysis of the Sarin-3-nitrocatecholate ester demonstrated both acid catalysis and dealkylation which are specific properties exhibited in the enzymic "ageing" reactions. The dealkylation of the isopropyl group was intramolecularly facilitated by the vicinal hydroxyl group of the 3-nitrocatechol moiety. Dealkylation of isopropanol has previously been reported for the "ageing" effect of cholinesterase

compounds. Only under extremely alkaline conditions(pH 10-13) was the cleavage reversed to yield 3-nitrocatechol as the leaving group. The product of the isopropyl cleavage was a mono-alkyl phosphonate ester which was resistant to further hydrolysis in the pH region 3-13. Only under extremely acidic conditions could this ester be hydrolyzed and the 3-nitrocatechol dephosphorylated.

The total pH profile was investigated and the reaction products identified in an attempt to determine the behavior of Sarin under conditions favoring intramolecular catalysis of its ester. The ester hydrolysis was studied as two separate reactions:

A) In the pH range 2-7, the dealkylation reaction accompanied by the loss of the isopropyl moiety seems to be internally acid catalyzed and promoted by a nucleophilic attack of water and other nucleophiles on the neutral ester species. The first order rate constant for this reaction at 30.0° was $k = 2.3 \times 10^{-2} min^{-1}$.

B) The 3-nitrocatechol was dephosphorylated only at extremely alkaline pH 10-13. A dianionic species of the ester is suggested as a possible intermediate to account for a saturation effect. The second

order rate constant for this reaction at 30.0° was $k = 2.9 \times 10^{-1}$ 1 $mole^{-1}min^{-1}$.

The study was also conducted at several temperatures and the apparent activation energies for the two proposed reactions were determined from the Arrhenius relationship to be: reaction A, 13.9 Kcal $mole^{-1}$ and reaction B, 14.3 Kcal $mole^{-1}$.

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